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13. ABSTRACT ***(Maximum 200 Words)***

This project explores the potential of a new and innovative approach to human gene therapy that may prove to be useful for the treatment or prevention of a range of genetic diseases including many types of cancer. We have previously demonstrated that a trans-splicing group I ribozyme can be employed to repair mutant transcripts in *E. coli* (Sullenger and Cech, *Nature* 1994) and mammalian cells (Jones et al., *Nature Medicine* 1996; Lan et al., Science 1998). Ribozyme-mediated repair of mutant mRNAs associated with a range of human diseases is now experimentally tractable, and we have begun to assess the therapeutic potential of this process for the repair of mutant transcripts implicated in the development and progression of breast cancer. Because mutation of the *p53* gene is the most common genetic change seen in a wide variety of malignancies including breast cancer, we have initially focused on repair of mutant *p53* transcripts. Toward this end, we have mapped the accessible sites for ribozyme binding on *p53* RNAs, and using this information we have generated ribozymes that can react with and repair mutant *p53* transcripts in breast cancer and other cell lines.

14. SUBJECT TERMS
RNA Repair, Trans-splicing Ribozyme, Gene Therapy, Suppressor Oncogene, p53

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Bruce A. Sullenger 9/6/99

PI - Signature Date

4. Table of Contents

5. Introduction

The overall goal of this project is to develop a new and innovative approach to human gene therapy that is based upon ribozyme-mediated repair of mutant mRNAs (Sullenger and Cech, 1994; Sullenger and Cech, 1995; Sullenger, 1995). This approach may be especially useful for the treatment of cancer because it should restore the regulated expression of oncogenes. Previously, we described how a trans-splicing ribozyme can be employed to repair mutant mRNAs in *Escherichia coli* (Sullenger and Cech, 1994). Subsequently we demonstrated that trans-splicing can also proceed in mammalians cells (Jones et al., 1996). Finally, we recently demonstrated that ribozymes could be employed to repair sickle ß-globin transcripts in erythrocyte precursors from patients with sickle cell disease (Lan et al., 1998).

Many mutant messenger RNAs from dominant and suppressor oncogenes have been identified that appear to be involved in tumorigenesis. Repair of any of these mRNAs may yield new insights into tumor development and treatment. However, we have initially focused upon the repair of mutant transcripts from the *p53* tumor suppressor gene. Much evidence indicates that loss of the p53 protein is associated with neoplastic transformation. Mutant *p53* transcripts are frequently found in a range of primary human tumors and tumor cell lines including mammary carcinomas (Nigro et al., 1989; Bartek et al., 1990; Malkin 1994). Thus we have sought to develop ribozymes that can repair the mutant p53 transcripts present in these tumor cells in hope of using such ribozymes to either revert the transformed phenotype of these cells or induce apoptosis. Toward this end the specific objectives of this research proposal have not changed and are:

1.) To determine which regions of mutant *p53* transcripts are accessible to trans-splicing ribozymes.
2.) To construct trans-splicing ribozymes that can repair mutant *p53* transcripts and test them in vitro.
3.) To evaluate ribozyme-mediated *p53* repair in tissue culture cells after transient transfection.
4.) To determine if the ribozyme can repair endogenous mutant *p53* transcripts in breast cancer cell lines harboring defective copies of the *p53* gene.
5.) To ascertain if repair of mutant *p53* transcripts renders breast cancer cells less tumorigenic.

6. Body

6.1. Overview

We have made significant progress toward fulfilling several of our specific aims since we received this grant on August 15th, 1997. One accomplishment is that we have determined which regions of the p53 transcript are accessible to trans-splicing ribozymes. Such mapping has been

performed upon p53 transcripts generated by in vitro transcription and mutant p53 mRNAs present in total cellular RNA isolated from MDA-MB-231 mammary carcinoma cells. These results fulfill our first specific aim: to map mutant p53 transcripts. In addition, we constructed two trans-splicing group I ribozymes that can recognize two of the most accessible regions on the p53 mRNA and have tested the ability of this ribozyme to react with the intended nucleotide on the targeted p53 transcript. Our results demonstrate that such trans-splicing ribozymes can react with p53 transcripts with high fidelity in the test tube as well as in MDA-MB-231 breast cancer cells. More recently, we have constructed plasmids containing ribozyme expression cassettes and have been able to demonstrate that trans-splicing ribozymes can repair mutant p53 transcripts in transiently transfected Saos-2 human osteosarcoma cells. These results partially fulfill our specific aims #2, #3 and 4: to create trans-splicing ribozymes that can repair p53 transcripts and test their activity in test tubes and in cells.

6.2 Studies and Results on Objective #1: To determine which regions of mutant *p53* transcripts are accessible to trans-splicing ribozymes (six months).

To ascertain which regions of the p53 transcript are accessible to ribozymes, we developed an RNA mapping strategy that is based on a trans-splicing ribozyme library and RNA tagging (Jones et al., 1996). To generate the mapping library, the guide sequence of the *Tetrahymena* group I trans-splicing ribozyme was randomized such that the 5' end of the RNAs in the library began with 5'-GNNNNN-3' where "G" represents guanine and "N" represents equal amounts of the 4 nucleotides. To map the p53 transcript in vitro, the mapping library was incubated under splicing conditions with either p53 transcripts generated by in vitro transcription using T7 RNA polymerase or with p53 transcripts present in total cellular RNA isolated from MDA-MB-231 breast cancer cells. To identify accessible uridine residues, the trans-splicing reaction products were reverse transcribed (RT) and amplified by the polymerase chain reaction (PCR) with primers specific for the ribozyme's 3' exon tag (Jones et al., 1996) and for the 5' end of the p53 target RNA. From this analysis, the uridines at positions 41, 65 and 384 of p53 RNA appeared to be particularly accessible in p53 transcripts regardless of whether the p53 transcripts were generated by in vitro transcription or isolated from MDA-MB-231 mammary carcinoma cells (Fig. 1). These mapping results, taken together with the fact that tumor cells often have mutations downstream of the nucleotide at position 360, encouraged us to focus on developing ribozymes that recognize the uridines present at positions 41 and 65 on the p53 mRNA. Thus, the internal guide sequence on the L-21 trans-splicing ribozyme was changed to 5'-GGAGGG-3' to generate a ribozyme, called Rib41, specific for site 41 and to 5'-GGGUCU-3' to generate a ribozyme, called Rib65, specific for site 65. In addition, inactive versions of these ribozymes, called Rib41d and Rib65d, which lack part of the catalytic core of the enzyme, were generated as controls (Sullenger and Cech, 1994).

Figure 1:Mapping Results of the Accessible Uridines on the p53 Transcript

reaction sites (nt)	number of clones	
	in vitro	cellular RNA from MDA-MB-231 cells
+24	49	-
+41	4	4
+65	2	2
+307	-	1
+332	-	1
+340	1	1
+384	6	5
+389	-	1

hot spots of mutations in human cancers

6.3 Studies and Results on Objective #2: To construct trans-splicing ribozymes that can repair mutant *p53* transcripts and test them in vitro (six moths).

Rib41 and Rib65 can trans-splice a 3' exon tag onto p53 transcripts in vitro. The trans-splicing ribozymes, Rib41-3'tag and Rib65-3'tag, were incubated under splicing conditions with p53 RNA generated by in vitro transcription or total RNA isolated from MBA-MB-231 cells. To determine if trans-splicing had occurred in any of the RNA samples, RT-PCR analyses were performed with one primer specific for the p53 target RNA and the other primer specific for the 3' exon tag sequence. An amplified fragment of the expected size was generated from samples containing Rib41-3'tag and Rib65-3' Tag and either in vitro transcribed p53 RNA or total RNA isolated from breast cancer cells (unpublished results). No such RT-PCR product was generated from samples that lacked a ribozyme or that contained the inactive versions of the ribozymes. Sequence analysis of the spliced products demonstrated that the ribozyme had reacted with the intended uridine. More recently, we have changed the 3'-exon attached to Rib41 so that it encodes the wild type sequence for the p53 transcript. Using this ribozyme, we have now been able to demonstrate that this ribozyme can repair p53 transcripts, that contain a point mutation at nucleotide 820, in the test tube (unpublished results).

6.4. Studies and Results on Objective #4: To determine if the ribozyme can repair endogenous mutant *p53* transcripts in breast cancer cell lines harboring defective copies of the *p53* gene (nine months).

To determine if Rib41-3'tag could react with mutant p53 transcripts inside human mammary carcinoma cells, the ribozyme was introduced into MBA-MB-231 cells via liposome-mediated transfection. RT/PCR amplification generated a fragment of the expected size (87 base pairs) from the total RNA isolated from the MBA-MB-231 cells that had been transfected with the active ribozyme (Fig. 2). By contrast no such product was generated from RNA samples isolated from cells that were not transfected or were transfected with the inactive ribozyme, Rib41d-3'tag. When Rib41-3'tag was added to the RNA extraction buffer used to isolate total RNA from a sample of mock transfected MBA-MB-231 cells, no amplification product was generated (Fig. 2) suggesting that the observed trans-splicing products were generated inside the transfected cells and not during RNA analysis. From these results we conclude that Rib41 can react with p53 transcripts inside human breast cancer cells. To confirm this result, we subcloned and sequenced a number of these PCR generated cDNAs shown in figure 2. The splice junctions on each of the 6 clones tested were identical and the sequence of a representative clone is shown in figure 3. This sequence analysis demonstrated that the ribozyme had reacted with the uridine at position 41 in the p53 transcript inside of these cells with high fidelity because in all 6 clones sequenced the 3' exon tag had been attached onto the proper uridine.

Figure 2: Amplification of p53 derived trans-splicing products

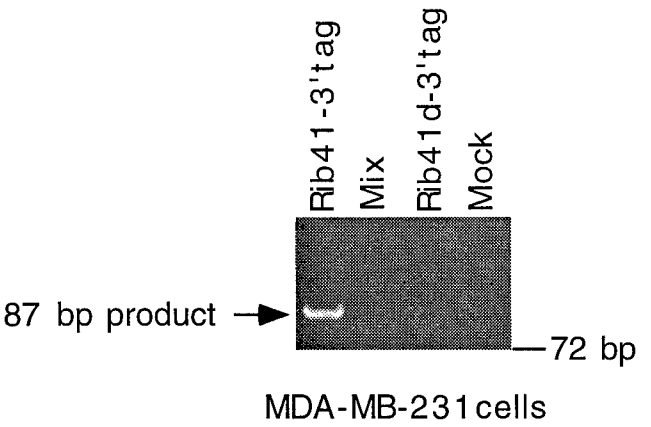
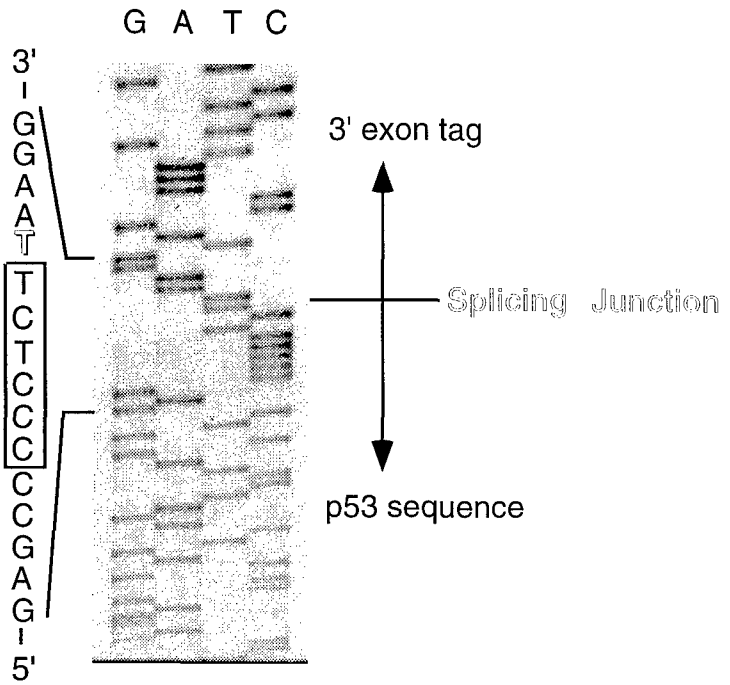
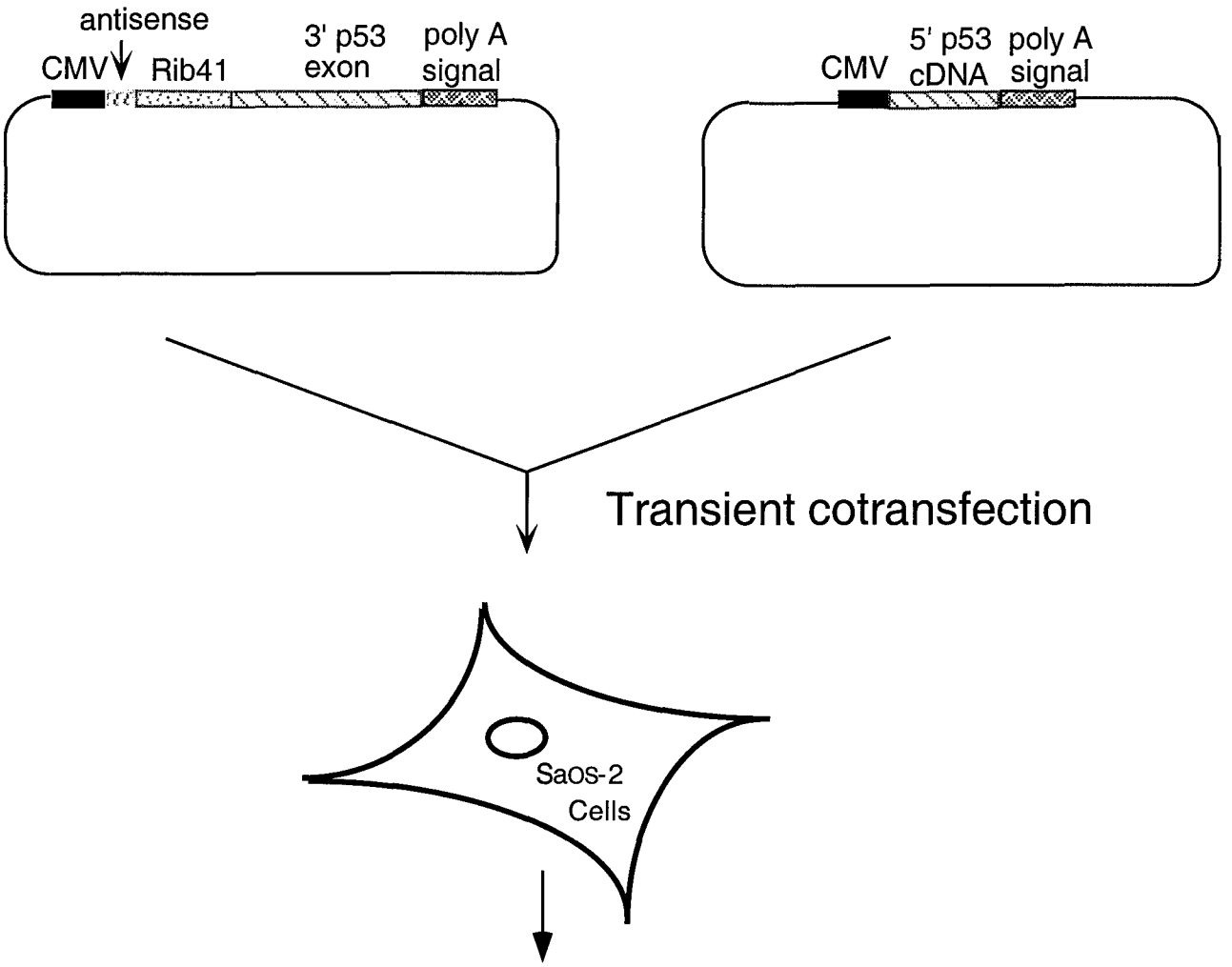


Figure 3: Sequence analysis of trans-splicing reaction products



MDA-MB-231 cells

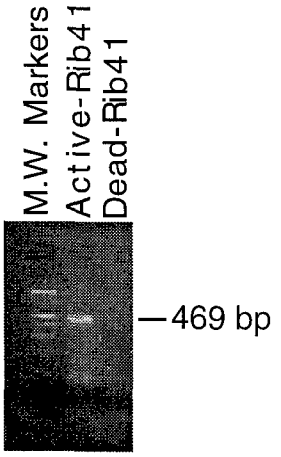
Figure 4: Ribozyme expression cassette design and evaluation



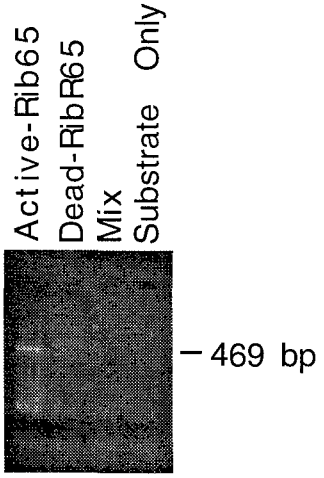
Isolate RNA and Analyze for Spliced Products

Figure 5: Trans-splicing mediated repair of p53 transcripts in Soas-2 cells.

A. Ribozyme 41



B. Ribozyme 65



6.5. Studies and Results on Objective #3: To evaluate ribozyme-mediated *p53* repair in tissue culture cells after transient transfection. (nine months).

To determine if Rib41 and Rib65 could repair mutant p53 transcripts following transisnet transfection, we first cloned the ribozymes into expression cassettes containing the CMV immediate early promoter and the preproinsulin polyadenylation signal sequence (Fig. 4). A number of expression cassettes were constructed that contained a variety of guide sequence lengths as well as the presence or absence of the P10 region of the ribozyme. Each of these ribozyme constructs were individually cotransfected with an expression cassette containing a truncated version of the p53 gene into Saos-2 human osteosarcoma cells which do not naturally containing p53 derived transcripts. Total RNA was harvested from these cells and analyzed for the presence of the repaired p53 transcript by reverse transcription and polymerase chain reaction techniques. As shown in figure 5, repaired RNAs could only be detected in RNA samples from cells expressing the active ribozymes and the truncated p53 substrate transcript; whereas, no repaired p53 RNA was detected in inactive ribozyme transfected cells.

7. Key Research Accomplishments

- Mapped accessible regions for ribozyme binding on p53 transcript
- Demonstated the trans-splicing ribozymes can react with mutant p53 transcripts in breast cancer cells.
- Demonstrated that RNA polymerase II expressed ribozymes can repair mutant p53 transcripts in Saos-2 cells.

8. Reportable Outcomes

None to date, but we intend on submitting a manuscript describing p53 RNA mapping and repair in the next few months.

9. Conclusions

In summary, we have made significant progress toward fulfilling several of our specific aims since we received the funding for this research proposal on August 15th, 1997. We have determined which regions of the p53 transcript are accessible to trans-splicing ribozymes using a novel RNA mapping strategy based upon a library of ribozymes and 3'-exon tagging. Such mapping has suggested that the nucleotides at positions 41 and 65 on the p53 transcript are particularly accessible to trans-splicing ribozymes. These results fulfill our first specific aim: to map the accessible regions on mutant p53 transcripts. In addition, we constructed two trans-splicing group I ribozymes that can recognize the uridines at positions 41 and 65 on the p53 mRNA and have tested the ability of these ribozymes to react with the intended nucleotide on the targeted p53 transcripts in the test tube and inside the human mammary carcinoma cell line MDA-MB-231. Our results demonstrate that these trans-splicing ribozymes can react with p53 transcripts with high fidelity in the test tube and that Rib41 can react with mutant p53 transcripts in breast cancer cells. More recently, we have genreated a number

of ribozyme expression cassettes and have been able to demonstrate that the ribozyme can repair truncated p53 transcripts following expression in cotransfected Saos-2 osteosacroma cells. These results partially fulfill specific aims #2, 3 and 4: to create trans-splicing ribozymes that can repair p53 transcripts and test their activity in test tubes and in cells. This next year, we plan to begin to assess what phenotypic effect(s) such p53 RNA repair has upon these breast cancer and other cells.

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